R89-18

Published in Agricultural & Food Chemistry, May/June 1989, 615–624, by the American Chemical Society

Use of Polyamide Oxidative Fluorescence Test on Lipid Emulsions: Contrast in Relative Effectiveness of Antioxidants in Bulk Versus Dispersed Systems[1]

William L. Porter,* Edward D. Black, and Anne M. Drolet

The polyamide fluorescence method of detecting malonaldehyde or precursors has been adapted to wet systems, using liposomes from sonicated soybean lecithin. Vapors arising from the oxidizing microdispersions produce fluorescent compounds with the end-group amines contained in a polyamide powder coated on plastic or glass. We have studied the hematin-accelerated reaction in covered Petri dishes with a polyamide strip in the vapor space at a temperature of 65 °C, using 25 mL of 0.013 M phosphate buffer, pH 5.5, containing 3 mg/mL sonicated lecithin. At pH 9.1 no fluorescence is measurable. The plate fluorescence intensity is measured with a solid sample holder in a fluorescence spectrophotometer. There is an excitation maximum at 360 nm and an initial emission maximum at 425 nm, shifting bathochromically with time. A series of antioxidants tested in the hematin-catalyzed system at 0.1% phospholipid display a largely reciprocal relation to their reported effectiveness in dry bulk oils, particularly vegetable oils. Further evidence for this "polar paradox" that nonpolar antioxidants function best in polar lipid emulsions and membranes while polar antioxidants are relatively more effective on nonpolar lipids is presented.

The military experience with lipids in foods has a wide range: storage of multipurpose cooking and salad oils, frying with these oils, storage and use in mayonnaise and salad dressing emulsions, comminuted and re-formed meats, baked goods, and freeze-dried items, to mention only a few. Oxidation of lipids, whether initiated by enzymatic, photooxidative, or metal catalysis, is a universal problem in shelf life of military rations. Antioxidants introduced to prevent or delay this process must be used in a wide variety of situations. Experience has shown that antioxidants' relative effectiveness varies widely. No general rationale for their use has been developed. Availability of such a protocol would result in large extensions of shelf life of military rations.

Porter has suggested (1980) that a useful rationale to simplify the many applications of primary antioxidants is that, ceteris paribus, compounds that are relatively polar, hydrophilic, or amphiphilic with high hydrophile–lipophile balance (HLB) number (Griffith, 1954; Adamson 1967) are relatively more effective in low surface to volume ratio (LSV) displays of lipid (bulk oils and fats, whether vegetable or animal, or synthetic esters of their constituent fatty acids). On the other hand, compounds that are relatively nonpolar, lipophilic, or amphiphilic with low HLB are relatively more effective in high surface to volume (HSV) lipid situations (emulsions, micelles, and membranes of whole tissue having colloidal dimensions, whether hydrated or dehydrated). The proposed rule is most applicable at the extremes of a continuum, i.e., in emulsions and membranes with a very low lipid phase concentration versus bulk vegetable oils. However, the proposed rule also generally applies to bulk animal fats and oils and to synthetic esters derived from these as well as to concentrated emulsions. Clearly, high volatility (BHA, BHT) or vulnerability to heat and alkaline conditions, as in the short-chain substituted hydroquinones and gallates, modifies the general trend, as do the introduction and processing conditions that emphasize these traits. Endogenous tocopherols also modify the effect in bulk vegetable oils.

Before 1955, applications of antioxidants and the tests for their relative effectiveness in stabilizing lipids were largely with dry bulk (LSV) fats and oils. Stability tests like the AOM test, the Schaal oven test, and the oxygen bomb test were most adaptable to bulk lipid, whether monitored by peroxide value (PV), pressure change, weight gain, or sensory change. A large body of relative effectiveness data for antioxidants was thus generated, and very often it has been extrapolated uncritically from bulk lipid (LSV) to HSV situations like emulsions, micelles, and

Department of the Army, U.S. Army Troop Support Command, Natick Research, Development & Engineering Center, Natick, Massachusetts 01760-5020.

[1] Presented at the meeting of the Division of Agricultural and Food Chemistry, 182nd National Meeting of the American Chemical Society, New York, NY, 1981.

membranes in whole tissue foods.

In more recent work, however, Chipault et al. (1956), Uri (1958, 1961), Simpson and Uri (1956), and Abbot and Waite (1962) commented on the contrast in relative effectiveness between the same antioxidant as used in bulk oils versus emulsions (or dehydrated emulsions like spray-dried whole milk). Berner et al. (1974) used heme-catalyzed lard emulsions monitored by polarography and noted the similarity of their results to those of Chipault. Scott et al. (1974), in Cort's group, found that decarboxylation or esterification of Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) resulted in increased antioxidant activity in a hemoglobin–safflower oil emulsion and greatly decreased activity in bulk oils. Trolox C is plainly more hydrophilic than its ester or decarboxylated product, since it is a relatively strong acid. Cort (1974, 1982) reported that ascorbic acid (AA) was much more effective than ascorbyl palmitate (AP) in bulk soybean oil and much less effective than AP in the hemoglobin–safflower oil emulsion test. Taylor et al. (1981) used polarography of hemoglobin-catalyzed methyl linoleate emulsions for testing amino acid residues covalently bound to Trolox C. He suggested that "the role of the solubility of a compound on its antioxidant activity in the linoleate emulsion deserves further study". Fukuzawa et al. (1982) found that tocopherols had a relative antioxidant effectiveness in egg lecithin liposomes of $\alpha > \beta > \gamma > \delta$, whereas the reverse order is known to hold in bulk edible oils (Moore and Bickford, 1952; Lea and Ward, 1959). The liposome order corresponds to the in vivo vitamin potency order (Century and Horwitt, 1965) and to the order of TLC elution in lipophilic solvents (Cillard and Cillard, 1980). Bieri et al. (1976) and Bunyan et al. (1960) also found α-tocopherol a much more effective antioxidant than γ-tocopherol against oxidative hemolysis of erythrocytes (HSV).

This reciprocal effect, the "polar paradox", is particularly pronounced in a homologous series like that of gallic acid and the alkylated gallate esters, where the most polar member, gallic acid, is the most effective in the dry oil AOM test (Morris et al., 1947; Sherwin, 1976; Thompson and Sherwin, 1966; Dziedzic and Hudson, 1984) and least effective in emulsions like those in baked goods (Morris et al., 1947). Similar effects appear in the caffeate and ferulate (Daniels and Martin, 1967; Dziedzic and Hudson, 1984), the flavone (Abbot and Waite, 1962; Simpson and Uri, 1956), and the hydroquinone series (Thompson and Sherwin, 1966). The extreme lipophiles BHA, BHT, and ethoxyquin, show a similar contrast, being relatively much more effective in emulsions than in dry lard or vegetable oils, as is shown by our present data and that of others (Lew and Tappel, 1956; Marco, 1968; Dziedzic and Hudson, 1984).

Liposomes (vesicles formed from dispersed phospholipids) are recognized as membrane models appropriate for pro- and antioxidant testing (Corliss and Dugan, 1970; Kaschnitz and Hatefi, 1975; Gutteridge, 1977; Barclay and Ingold, 1980; Kornbrust and Mavis, 1980; Koreh et al., 1982; Weenen and Porter, 1982; Wu et al., 1982).

Since Porter's review of food applications of antioxidants (1980), other workers have used colloidal dispersions of fatty acids for pro- and antioxidant evaluation. These are less direct membrane models than liposomes, and hence, the results are less applicable to whole tissue foods like meats or food-derived emulsions like mayonnaise, baked goods, or dehydrated whole milks. Emulsions of linoleic acid with various synthetic surfactants in buffer, catalyzed by either heme or enzyme or by autocatalysis, have been largely employed. For example, Lizada and Yang (1981) used diene conjugation to follow sulfite-induced peroxidation in linoleic acid emulsions buffered at pH 7 and its inhibition by antioxidants. Lipophilic butylated hydroxytoluene (BHT) at 3.7 μM gave the same inhibition as the hydrophilic hydroquinone (HQ) at 75 μM, a 20-fold difference.

For testing the relative effectiveness of antioxidants in both dry oils and lecithin liposomes and emulsions, we have used a fluorescence-producing reaction that we have found between the volatiles from oxidizing lipids and polyamide powder in thin films on solid supports (Porter, 1981; Porter et al., 1980, 1981, 1983). The fluorescence has some features (Porter et al., 1983) comparable to those resulting from the combination of malondialdehyde and primary amines (Chio and Tappel, 1969). It can be quantified by solid sample fluorescence spectrophotometry, using the methods of Sawicki et al. (1964) and Guilbault (1977).

In the present work, polyamide fluorescence has been used to assay the relative effectiveness of antioxidants in oxidizing soy lecithin liposomes and emulsions. Results are contrasted with those in bulk vegetable oils reported in the literature.

MATERIALS AND METHODS

For fluorescence assessment of the vapor phase over oxidizing lipid emulsions, we have used a sonicated aqueous dispersion (3 g/L) of either crude soybean lecithin (Yelkin, DS, 35% acetone solubles, mostly triglyceride) or acetone-extracted lecithin (3–5% acetone solubles). These were procured from Ross and Rowe, Inc. The crude lecithin microdispersion was largely used for the work reported here. The microdispersion is cloudy gray and has spherical particles ranging from 0.1 to 1.2 μm, the most frequent particle size being about 0.1 μm. The acetone-stripped lecithin (Arlec) is in dry granular form and requires only 5-min sonication to produce a clear, opalescent microdispersion with no particles visible at 1250× magnification (optical microscope).

The crude lecithin has been double-bleached in factory processing with hydrogen peroxide and benzoyl peroxide. Ultraviolet spectra of the phosphatide microdispersions prior to adding the accelerator hematin showed no measurable conjugated diene absorption at 233 nm. Sonicated microdispersions were prepared either the day of an experiment or the day before and stored overnight at 40 °C in the dark.

Tests of relative effectiveness of antioxidants were conducted with hematin acceleration. Hematin was procured from Calbiochem-Behring Corp. It is used as received, since in our tests the UV spectrum and TLC behavior indicate purity.

The antioxidants methyl gallate (MG), ethyl gallate (EG), propyl gallate (PG), octyl gallate (OG), and dodecyl gallate (DG) were donated as samples by NIPA Laboratories, Ltd., Mid Glamorgan, Great Britain. Topanol 354 (2,6-di-*tert*-butyl-4-methoxyphenol, TOP) was donated as a sample by Petrochemicals Division, Imperial Chemical Industries, Ltd., Teesside, England. Poly AO 79 (POLY) was donated as a sample by Nicolo Bellanca, Dynapol, Inc., Palo Alto, CA. BHA and TBHQ were food-grade compounds supplied by Eastman Chemical Products, Kingsport, TN. BHT was food-grade and was donated as a sample by Koppers Co., Inc., Pittsburgh, PA. Gallic acid (GA) and quercetin (Q) were procured from Pfalz and Bauer, Inc., caffeic acid (CA) from J. T. Baker Chemical Co., and chlorogenic acid (CHLA) from Aldrich Chemical Co. Ethoxyquin (Santoquin = SAN) was donated as a sample by Monsanto Chemical Co. Hydroquinone (HQ) was procured from Mallinckrodt Chemical Works.

Antioxidants were tested for purity by melting point and thin-layer chromatography (TLC). TLC was carried out on heat-activated silica with solvent systems (1) chloroform, (2) chloroform/methanol (19/1), and (3) chloroform/methanol/acetic acid (19/1/0.1).

A Hitachi solid sample holder attachment for Model MPF-2A Hitachi Perkin-Elmer fluorescence spectrophotometer was used in the research reported here. Sonication was accomplished with a Biosonik BP-III Ultrasonic System, Bronwill Scientific. A solid

sample holder designed by personnel of Baird Corp. and the Baird Atomic Model SF-1 fluorescence spectrophotometer are used for cooking and salad oil shelf life testing and for antioxidant testing in dry oils.

Characteristics of the polyamide substrate and the fluorescence-producing reaction have been described (Porter et al., 1983) as have the details of measurement of plate surface fluorescence by means of a solid sample holder. Essentials of the standardized method and of lipid emulsion preparation are summarized below. Plate fluorescence may be measured within or in the vapor space above an oxidizing oil or within an aqueous emulsion of lipid and if the pH is below approximately 5.5, in the vapor space above it. It might be noted that pK_a of malonaldehyde is 4.65 (Kwon and Watts, 1964).

The lecithin is dispersed in deionized water containing 0.013 M phosphate buffer, pH 5.5. The crude, plastic lecithin is sonicated at maximum power for 20 min. Sonication is carried out under a stream of nitrogen, with the vessel suspended in an ice–salt bath.

In the standard preparation, 75 mg of hematin is dissolved in 75 mL of deionized water with 8 drops of 10% KOH and brought to a volume of 100 mL. Of this preparation, 2 mL is added to 50mL of the microdispersions at zero time to give a phosphatide hematin ratio of 100/1. Final pH is 5.5–5.6.

For emulsion tests, polyamide-coated terephthalate plastic plates are attached powder side down by double-faced transparent tape strips to the undersurface of the lid of 9 × 1 cm Pyrex Petri dishes. The plates, 2 × 3 cm, are cut from standard 20 × 20 cm polyamide–terephthalate plates used for thin-layer chromatography. They are Polygram Polyamide-6 UV_{254}, procured from Macherey-Nagel Co. through Brinkmann Instruments, Inc., Westbury, NY. As indicated, they contain a fluorophore (zinc silicate) activated by short-wave UV but are not active in the 360-nm range used herein. For the usual test, 25 mL of microdispersion is placed in the dish, about 4 mm in depth.

In the standard method, antioxidants are added at 0.1% by weight of dispersed lipid. In a typical test, 50 mL of the lecithin microdispersion is treated with 0.5 mL of ethanol containing 0.15 mg of antioxidant. Controls are 50 mL of dispersion and 0.5 mL of ethanol. After addition, the dispersions are bubbled with glass-filtered air for 30 min. Two milliliters of hematin solution is added at zero time to each 50-mL portion and 25 mL of the mixture placed in each Petri dish. The covered dishes, with attached polyamide strip, are placed in a 65 °C draft oven and sampled at 30-min intervals by removing the dish to a room-temperature cupboard, substituting a labeled Petri lid for the lid with polyamide strip, and measuring the accumulated fluorescent material in the solid sample fluorescence spectrophotometer, a process taking 5–8 min.

RESULTS

We have attempted to model the oxidation of a typical food emulsion (shortening in baked foods, salad dressing, or dried milk) by using sonicated microdispersions of crude soybean lecithin. We have also attempted to model the oxidation of membranes, e.g. erythrocytes, by using a sonicated microdispersion of acetone-extracted crude soybean phospholipids as did Kaschnitz and Hatefi (1975) in elegant work with various heme catalysts and superoxide anion.

Characteristics of the Lecithin. Thin-layer chromatography of the crude lecithin on silica gel using chloroform as eluant reveled two main spots, the phosphatides at the origin and the triglycerides at R_f 0.67 (sterol esters and pigments contaminate the latter spot). Complete acetone extraction removes all the contaminants to leave only phosphatide. In the commercial acetone-stripped lecithin (Arlec) that we have used, this process is incomplete and the material is only 95% acetone insoluble.

Our previous GC analyses of the transmethylated phosphatides from this material, commercial soybean "lecithin", have shown 54% linoleic acid and 5% linolenic acid. The material designated soybean "lecithin" of course contains not only phosphatidylcholine but also substantial amounts of phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid. Both of the latter confer a substantial negative charge on the microdispersed particles.

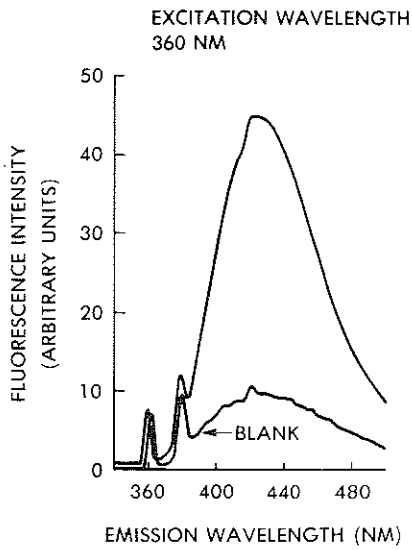

Figure 1. Oxidative polyamide fluorescence spectrum of oxidized soy lecithin emulsion, 65 °C.

We have used both the commercial crude and stripped materials without further purification in tests of relative antioxidant effectiveness because they effectively simulate two different food exposures of phosphatide. The crude material simulates a typical emulsified oil (35% triglyceride), as in emulsified baking shortening, milk fat globule, or salad dressing, while the stripped powder in microdispersion (3–5% triglyceride) is an approximate membrane model (as in erythrocytes), (Kaschnitz and Hatefi, 1975).

Although the crude material has 35% acetone solubles, we found only traces of compounds that react with the Emmerie–Engel reagent, a test for potential antioxidants, particularly tocopherols. In particular, no spot was found for α-tocopherol. This is to be expected, since the product has been peroxide treated in bleaching. Tocopherols are largely removed from the stripped lecithin by the acetone extraction.

Characteristics of the Fluorescence Spectra. Figure 1 shows a typical fluorescence spectrum produced on polyamide powder exposed to the vapors of an oxidizing aqueous soy lecithin microdispersion at pH 5.6 and 65 °C. Similar spectra are obtained from oxidizing polyunsaturated fatty acids, their esters, and triglycerides in emulsified or dry form and from such foods as potato chips and dried carrots. In this case, excitation maximum is 360 nm and emission 425 nm. The blank is the spectrum from a polyamide plate exposed similarly but without lipid. There is a residual scatter peak at 360 nm not completely removed by the customary 390-nm filter and a pattern of diffraction peaks produced by the polyamide particles (and indeed by any fine-powder-coated surface like silica TLC plates). The blank pattern has been discussed before (Porter et al., 1983). It is gradually obliterated by the increase in oxidative fluorescence.

The residual scatter peak at 360 nm is used as an effective relative internal reference. Thus, we define fluorescence index (FI) as the ratio I(emission peak)/I(scatter peak). In the lecithin work and especially in early stages of oxidation this approximates I(430 nm)/I(360 nm), but in later stages, emission shifts to progressively longer wavelengths. This is measured at every sampling period

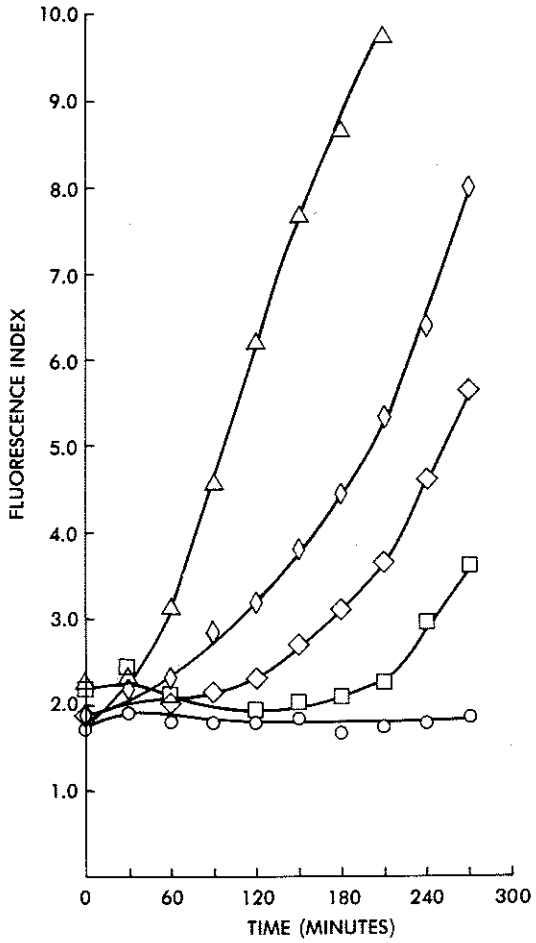

Figure 2. Effect of hematin concentration on oxidative polyamide fluorescence from soy lecithin emulsions, 65 °C: (○) blank; (□) lecithin; (△) lecithin plus hematin, 47 μM; (◇) lecithin plus hematin, 4.7 μM; (◇) lecithin plus hematin 0.47 μM.

for the blank, the control without antioxidant, and the antioxidant-treated sample. Relative mean deviation of replicate measurements (repositioning the plate) is ±5%.

If fluorescence index is plotted against time, there is always a typical autoxidative induction period, however short, either with or without antioxidant in the system. The antioxidant-dependent induction periods may be very long in microdispersions and the transition to rapid phase oxidation abrupt. Prooxidants like hematin (emulsion) or cobalt (dry oil) accelerate and antioxidants inhibit or slow the development of fluorescence. As noted before (Porter, 1983), fatty acids and esters with only two methylene-interrupted double bonds (linoleic acid) oxidize and produce fluorescence at a lower rate than the linolenate of soybean phosphatides. Also, the fluorescence is somewhat broadened toward the greenish yellow, whereas the initial soy emission is pure blue.

Kinetics. A plot of fluorescence index versus time for lecithin dispersions with and without added hematin at various concentrations appears in Figure 2. Without hematin, no plate fluorescence develops for 3 h, an induction period largely dependent, it would seem, on traces of endogenous antioxidants like the tocopherols. No rancid odor is detectable during this period, but it always develops rapidly and simultaneously when plate fluorescence appears. Similarly, the pale tan of the hematin-containing dispersion persists until the beginning of rapid fluorescence development, after which it rather rapidly decolorizes, both visibly and spectrophotometrically with loss of the typical 607-nm hematin absorption.

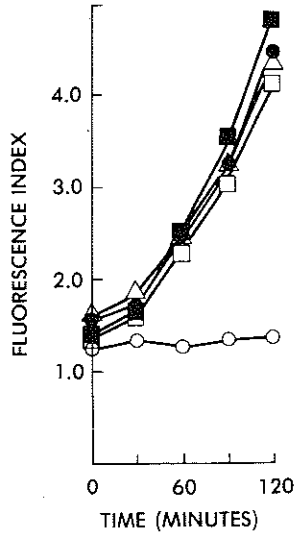

Figure 3. Effect of age of hematin solution on oxidative polyamide fluorescence from soy lecithin emulsions, 65 °C: (○) blank; (□) 69 days; (△) 14 days; (●) 7 days; (■) new solution.

Hematin greatly accelerates fluorescence development until, at the highest concentration, 47 μM, a very sharp break to a very nearly linear high rate occurs. This appears to be the phenomenon of maximum rate (Labuza, 1971), since the rate gives the appearance of zero-order kinetics for nearly the full extent of fluorescence development. Fluorescence index may range from just over 1 for the blank to values as high as 15–20. The least observable difference is between 1 and 2% of maximum range. Relative mean deviation of replicate measurements (repositioning the plate) is ±5%. We observed no inhibition at the highest hematin concentrations, contrary to the observations of others (Kaschnitz and Hatefi, 1975; Kendrick and Watts, 1969). Molar ratio of fatty acid/hematin for maximum catalysis was found to be 100 by Kendrick and Watts. Our estimated ratio is about 60/1.

Figure 3 shows that the age of the hematin solution has a small effect on the hematin-accelerated fluorescence development, older solutions being somewhat less effective. We have found hematin age to have more effect on induction period than on rate when added antioxidants are used.

Findings on the effect of age of the lecithin stock from which microdispersions were prepared (either on the day of or the day before the experiment) appear in Figure 4. Again, the older stock, containing presumably more hydroperoxide, reaches rapid phase first, although both stocks have good shelf life and show no measurable 233-nm absorption. Again, we have found that the age of the lecithin stock has a more pronounced effect on induction period than on rate when added antioxidants are being tested. This is especially true with crude lecithin, which requires 20 min of sonication, an oxidatively aggressive procedure.

Figure 5 shows the effect on rate of a total acetone extraction that we performed on the crude lecithin. The induction period for the totally stripped lecithin microdispersion is about half that for the crude bleached lecithin. This is presumably due to the depletion of the already low endogenous tocopherols, since on TLC the totally stripped lecithin shows no nonpolar spots for triglycerides or nonpolar compounds. The tocopherols would be expected to be acetone-extracted with the triglycerides.

The effect of fluorescent light on the fluorescence development is displayed in Figure 6. We were concerned that having samples periodically stand at room tempera-

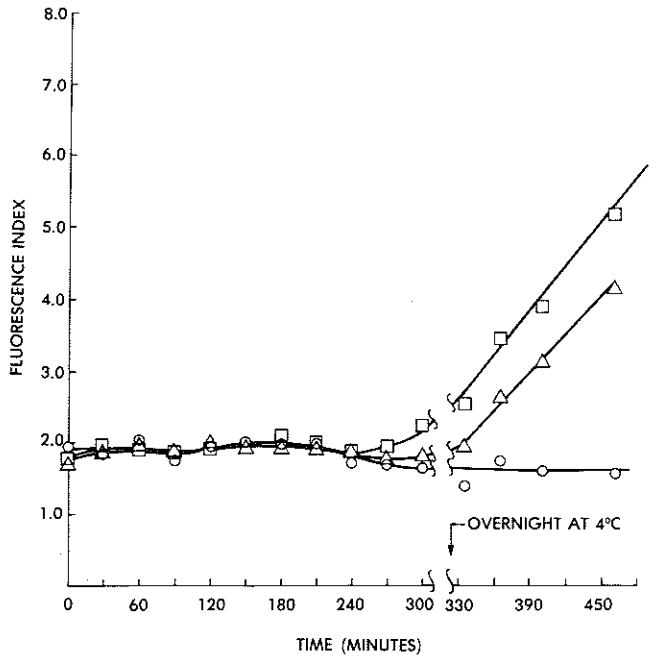

Figure 4. Effect of age of lecithin on oxidative polyamide fluorescence from soy lecithin emulsions, 65 °C, no hematin: (○) blank; (□) lecithin used 1 year after procurement; (△) lecithin used 1 month after procurement.

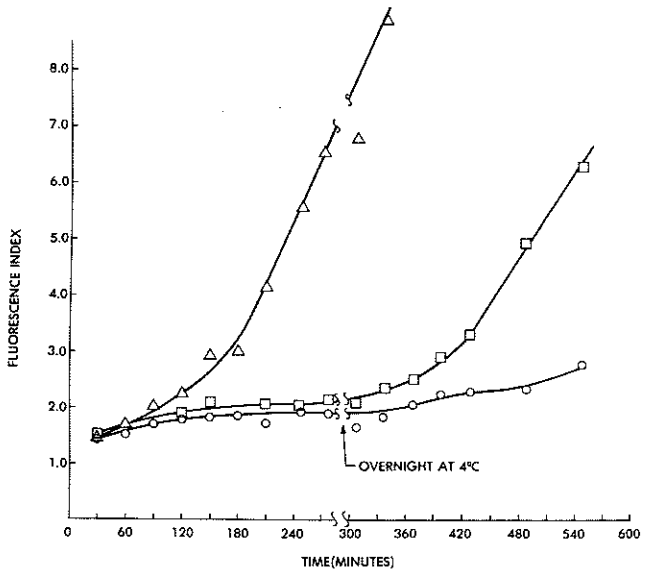

Figure 5. Effect of acetone extraction of crude lecithin on oxidative polyamide fluorescence from soy lecithin emulsions or liposomes, 65 °C: (○) blank; (□) crude lecithin; (△) acetone-extracted lecithin.

ture in diffuse fluorescent room light during the recurring (30-min) measurements of fluorescence on the sampling plate, a process itself of about 5–8 min, would accelerate sample oxidation and fluorescence. There seems to be little effect shown.

Figures 7 and 8 show results with two antioxidants—a lipophile, BHA, and a polar flavonol, quercetin. Clearly, the longer induction period in emulsions belongs to BHA. For quantification of this work, we use a protective index, called in our case relative effectiveness (REFF). It is defined as $(IP_A/IP_C) - 1$, where IP_A is induction period with added antioxidant and IP_C is induction period without added antioxidant.

For our purposes, we defined induction period graphically as the number of minutes between zero time and the

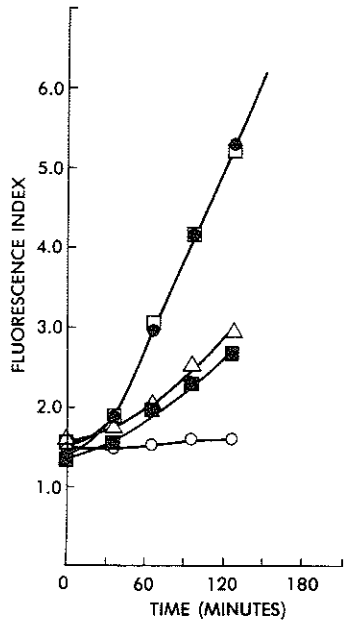

Figure 6. Effect of light on oxidative polyamide fluorescence from soy lecithin emulsions, 65 °C: (○) blank; (□) dark, 47 μM hematin; (△) dark, 4.7 μM hematin; (●) light, 47 μM hematin; (■) light, 4.7 μM hematin.

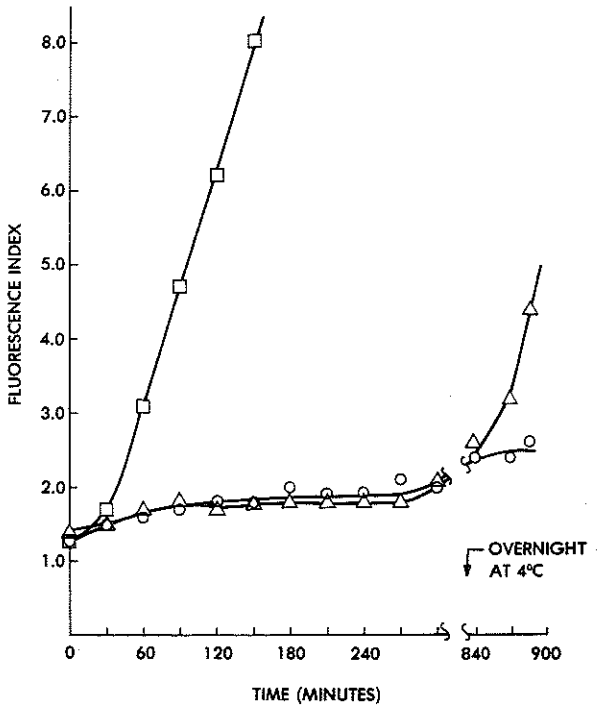

Figure 7. Antioxidant evaluation by oxidative polyamide fluorescence from soy lecithin emulsions, 65 °C: (○) blank; (□) lecithin plus hematin; (△) lecithin–hematin plus 0.1% BHA.

intersection of the extension of the tangent to the linear portion of the curve corresponding to rapid phase fluorescence development with the curve for the blank.

Using these methods we have quantified the relative effectiveness of some 16 antioxidants, both in individual experiments and in conjunction with a common repeated control substance like BHA. The number of experiments was 71; the average was 5 and was always greater than 3 for a given antioxidant, and can be as high as 12 for critical compounds, or those with high variability.

Figures 9 and 10 show the structures of the antioxidants tested. The extremely lipophilic monohydric phenols are

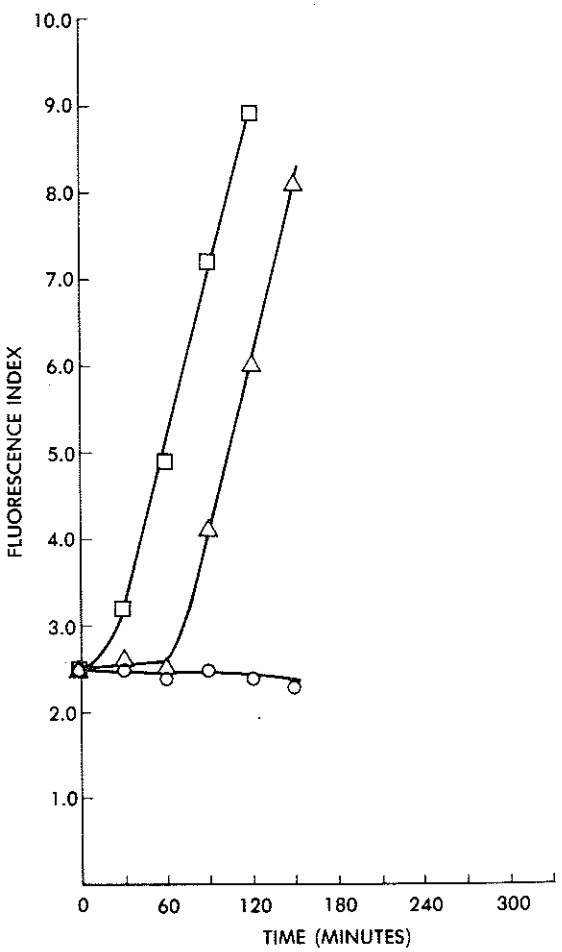

Figure 8. Antioxidant evaluation by oxidative polyamide fluorescence from soy lecithin emulsions, 65 °C: (○) blank; (□) lecithin plus hematin; (△) lecithin–hematin plus 0.1% quercetin.

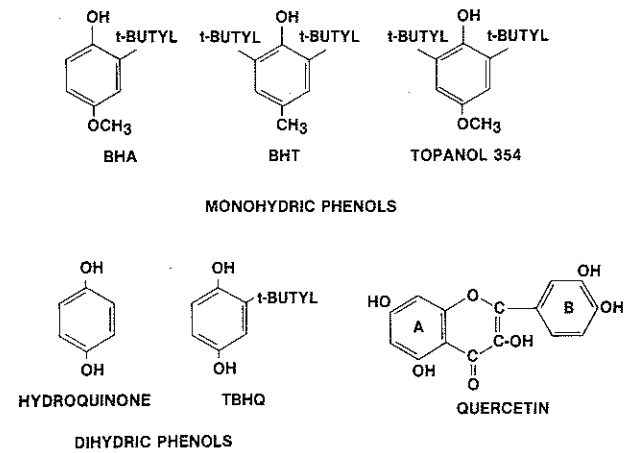

Figure 9. Monohydric and dihydric phenols.

shown in Figure 9. Steric hindrance of the aryloxide or arylamine radical is the great stabilizer of these compounds although resonance contributes. Figure 9 also shows the more polar para dihydric phenols derived from hydroquinone and the flavonol quercetin. Mesomeric resonance in the semiquinone radical stabilizes these. The trihydric compounds derived from pyrogallol, gallic acid, and the gallates appear in Figure 10. Mesomeric resonance of the semiquinone radical stabilizes these also. Gallic acid has a pK of 4.4 and would be largely anionic at pH 5.6. Figure 10 also shows the ortho dihydric phenolic acid, caffeic acid, and its very polar quinic acid ester chlorogenic acid. The

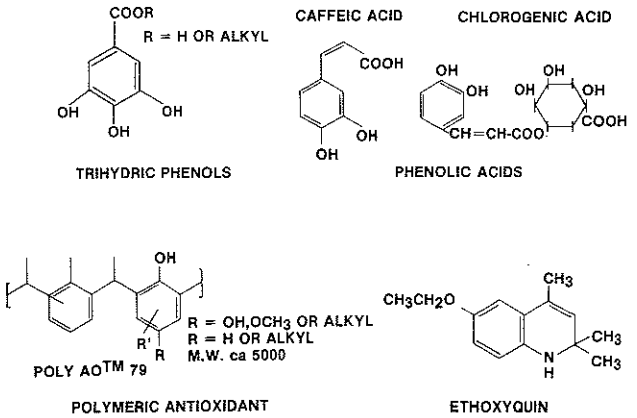

Figure 10. Trihydric phenols, phenolic acids, ethoxyquin, and polymeric antioxidant.

Table I. Relative Effectiveness of the Most Effective Antioxidants in Soy Lecithin Emulsions: REFF = IP_A/IP_C

compound	mean[a]	std dev	coeff of variation
BHA	32	±7.8	0.24
BHT	21	8.1	0.39
ethoxyquin (SAN)	13	5.6	0.43
propyl gallate	11	3.8	0.35
octyl gallate	11	7.9	0.72
dodecyl gallate	10	6.0	0.60
topanol	10	6.3	0.63
ethyl gallate	7	5.8	0.78

[a] Highest three values.

Table II. Relative Effectiveness of the Least Effective Antioxidants in Soy Lecithin Emulsions: REFF = IP_A/IP_C

compound	mean[a]	std dev	coeff of variation
TBHQ	4.9	±4.4	0.90
quercetin	4.1	1.7	0.41
methyl gallate	3.2	0.4	0.13
hydroquinone	3.2	0.9	0.28
gallic acid	1.4	0.3	0.21
gaffeic acid	1.4	0.2	0.14
chlorogenic acid	1.4	0.1	0.14
Poly AO 79	1.1	0.2	0.18

[a] Highest three values.

pK's are similar to those of gallic acid. Also shown is the new polymeric antioxidant developed by Dynapol, Inc., Poly AO 79. It is a very large molecule of 5000 molecular weight that is not absorbed from the gut. The extremely lipophilic aromatic monoamine ethoxyquin is sterically stabilized.

Table I displays relative effectiveness of the eight most effective antioxidants in soy lecithin microdispersions. Table II shows the eight least effective antioxidants. Means and standard deviations in this table are for the three highest REF's recorded for each antioxidant. The high coefficient of variation for some of the alkylgallates, topanol and TBHQ, is unexplained. REFF for these tables is IP_A/IP_C.

Figure 11 shows in graphic form the mean relative effectiveness of our antioxidants in both soy lecithin microdispersions and, in contrast, in the AOM in dry oils as reported in the literature (Table III). Means for this figure were calculated from *all* available trials in all cases except topanol for which two pilot experiments were rejected, since the method was not yet standardized. It is plain that there is a roughly reciprocal relationship, i.e., polar antioxidants are more effective in nonpolar environments with

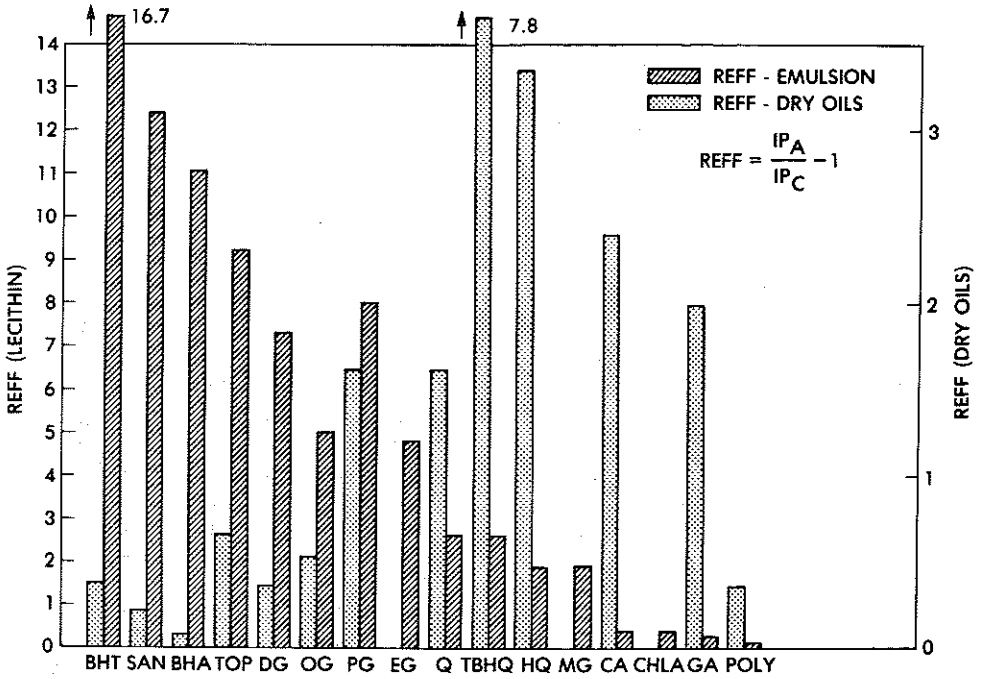

Figure 11. Relative effectiveness (REFF) of antioxidants in soy lecithin and dry vegetable oils: BHT, butylated hydroxytoluene; SAN, ethoxyquin (Santoquin); BHA, butylated hydroxyanisole; TOP, topanol 354; DG, dodecyl gallate; OG, octyl gallate; PG, propyl gallate; EG, ethyl gallate; Q, quercetin; TBHQ, tertiary butylhydroquinone; HQ, hydroquinone; MG, methyl gallate; CA, caffeic acid; CHLA, chlorogenic acid; GA, gallic acid; POLY, polymeric antioxidant (POLY AO 79). Values shown are means of all trials. REFF = $(IP_A/IP_C) - 1$. Note different scales for emulsions and dry oils.

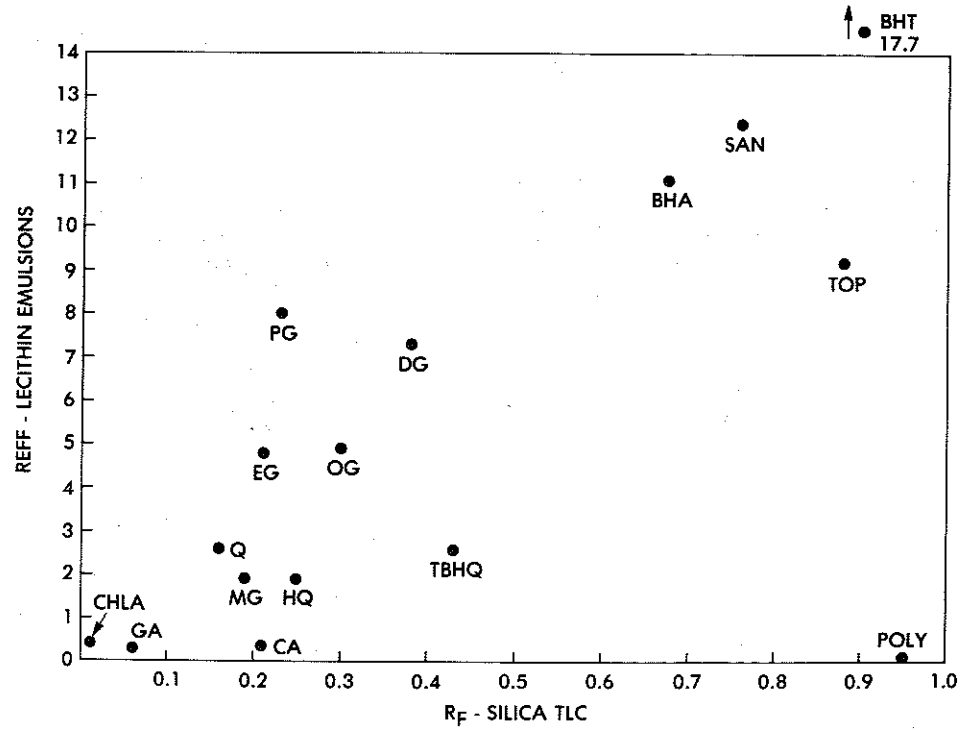

Figure 12. Relative effectiveness (REFF) of antioxidants in soy lecithin emulsions versus TLC mobility. R_f measured on silica in chloroform/methanol/acetic acid (19/1/0.5). REFF = $(IP_A/IP_C) - 1$. Abbreviations correspond to these of Figure 11.

nonpolar lipids (dry vegetable oils) and vice versa although there are notable exceptions, like Poly AO 79, which has no activity in our lecithin microdispersions. Figure 12 shows the antioxidants' relative effectiveness plotted against a rough measure of polarity, the R_f value in silica gel TLC chromatography using chloroform/methanol/acetic acid (19/1/0.5) as solvent. Again, the relationship is not rigidly linear, but the trend is there; i.e., nonpolar antioxidants are more effective in polar lipid emulsions. We have not used statistical analysis, because we are planning to use a more refined system for evaluating partitioning between dispersed lipid phase and continuous phase.

Chemical Characteristics of the Reaction. Chemical characteristics of the oxidative fluorescence-producing reaction on polyamide powder have been discussed (Porter, 1983). Some of the evidence is superficially consistent with but not sufficient to establish the involvement of vapor

Table III. Literature Sources Consulted for Values of Antioxidant Relative Effectiveness in Bulk Dry Oils[a]

antioxidant	substrate	source
BHT	safflower oil	Thompson and Sherwin, 1966
BHT	lard	Moore and Bickford, 1952
	cottonseed oil	
	hydrogenated cottonseed oil	
SAN	safflower oil	Thompson and Sherwin, 1966
BHA	safflower oil	Thompson and Sherwin, 1966
BHA	lard	Moore and Bickford, 1952
	cottonseed oil	
	hydrogenated cottonseed oil	
TOP	sunflower seed oil	Petrochemical Division ICI, 1974
DG, OG, PG	cottonseed oil	Sherwin, 1976
PG	safflower oil	Thompson and Sherwin, 1966
PG	lard	Moore and Bickford, 1952
	cottonseed oil	
	hydrogenated cottonseed oil	
PG	oat oil	Daniels and Martin, 1967
Q	ethyl linoleate	Geissman, 1962
TBHQ	safflower oil	Thompson and Sherwin, 1966
HQ	safflower oil	Thompson and Sherwin, 1966
HQ	lard	Moore and Bickford, 1952
	cottonseed oil	
	hydrogenated cottonseed oil	
CA	oat oil	Daniels and Martin, 1967
GA	safflower oil	Thompson and Sherwin, 1966
POLY	cottonseed/soybean oil blend	Furia and Bellanca, 1977

[a] Abbreviations correspond to the text and Figure 11.

phase malonaldehyde. It is of note that Kikugawa and Ido (1984) have found evidence from their system that the lipofuscin fluorophore does not seem to originate from a malonaldehyde–amine reaction. In their system (neutral pH), malonaldehyde produces fluorophores of longer excitation and emission wavelengths and different responses to chemical stress than those found for previous lipofuscin models (Chio and Tappel, 1969). We have similar data, not reported here, that tentatively support the production of polyamide fluorescence by volatile oxidation products other than malonaldehyde, possibly monofunctional enals or dienals.

DISCUSSION

The Polar Paradox Rationale. In early antioxidant testing as noted above, there was much more emphasis on the use of bulk oils and fats with a low surface to volume ratio (LSV) than on colloidal displays like emulsions, liposomes, and membranes of high surface to volume ratio (HSV).

Porter (1980) summarized the results of dry oil (LSV) tests and emulsion and ultra-thin-layer tests (HSV) as the polar paradox; i.e., primary antioxidants that are nonpolar or are amphiphiles of low HLB tend to be relatively more effective in polar emulsions (HSV) and with polar lipids, whereas primary antioxidants that are polar or are amphiphiles of high HLB tend to be more effective in dry oils (LSV), a nonpolar medium.

Our present results for the most part confirm this. The lipophilic antioxidants tend to be more effective in the emulsion or liposome system and less effective in bulk dry oils (Figure 11; Table III). The reverse is generally true for the more polar antioxidants (or amphiphiles with higher HLB). Thus, in the members of a homologous series like the gallates we show largely a reciprocal relationship: the lower (less alkylated) members tend to be more active in dry oils and the higher members more active in emulsions. When compounds not in a homologous series are compared, innate potency may differ but the more lipophilic compounds tend to be more effective in the emulsion (or liposome) system and vice versa. This confirms the results of others mentioned above and more recent results using amino acid derivatives of Trolox C (Taylor et al., 1981) and the tocopherols (Fukuzawa et al., 1982; Bieri et al., 1976; Bunyan et al., 1960).

The superiority of polar antioxidants and amphiphilic antioxidants with high HLB in bulk dry oils is most pronounced in bulk vegetable oils, which, of course, contain residual tocopherols. However, the superiority is present to a lesser degree in animal fats like lard (Moore and Bickford, 1952; Morris, et al., 1947; Dziedzic and Hudson, 1984) and in purified fatty acid esters with no detectable tocopherols (Lea, 1960). In very rapidly oxidizing esters like those from cod liver or linseed oil, the effect is much reduced, but still present (Lea, 1960; Olcott and Einsett, 1958). This may be related to the extreme antagonism Olcott and Einsett found in fish oils (low tocopherol) between added tocopherol and lipophilic antioxidants, but not against relatively polar ones like propyl gallate (Olcott and Einsett, 1958).

The relative ineffectiveness of more polar antioxidants in emulsions and liposomes (high surface to volume ratio) has significance in the food application of antioxidants derived from natural sources. Except for the tocopherols these are preponderantly polar compounds (phenolic acids, flavonoids, catechins, hydrolyzable and condensed tannins), and at equimolar concentrations can be expected to be relatively ineffective in the membranes of whole tissue foods and in emulsified foods (dried milks, bakery products, and convenience foods) compared to lipophilic or low HLB amphiphilic compounds. Where they occur at very high concentrations in foods, as in vegetarian seed and fruit regimes (Pratt and Miller, 1984) or tea, coffee, and wines (Sanderson et al., 1976), they can be expected to be more effective, especially when tested at very high concentrations with respect to the oxidizable lipid (Pratt, 1976).

However, at these concentrations, the undesirable effects of enzymatic browning, coloration with trace metals, and complexation of essential amino acids, proteins, and thiamine may also occur (Sosulski and Fleming, 1977).

There are few promising natural sources of lipophilic or low HLB antioxidants. Daniels and Martin (1967) have found high concentrations of long-chain alkyl esters of caffeic and ferulic acids in shelled oats. Sterol and triterpene alcohol esters of caffeic acid have recently been isolated from canary seed in rather high concentration (Takagi and Iida, 1980). These esters have the advantage that they are hydrolyzed to caffeic or ferulic acid and long-chain fatty acids or sterols, all familiar compounds in the gut of omnivores. Carnosol, isolated in pure form from rosemary leaves by Chang's group (Wu et al., 1982), is very effective in lard and since it is lipophilic, should be more effective in emulsions, providing the bulky polycyclic ring system is sterically compatible. Wenkert et al. (1965) show evidence, however, that carnosol is an artifact of extraction and that the naturally occurring parent substance is the more polar carnosic acid.

ACKNOWLEDGMENT

Thanks are extended to NIPA Laboratories, Ltd., for a gift of five alkyl gallates; to Petrochemicals Division, Imperial Chemical Industries, Ltd., for a gift of Topanol 354; to Nicolo Bellanca, Dynapol, Inc., for a gift of Poly AO 79; to Eastman Chemical Products, for a gift of BHA and TBHQ; to Koppers Co., Inc., for a gift of BHT; and to Monsanto Chemical Co., for a gift of santoquin (ethoxyquin). The secretarial assistance of Patricia Crawford is gratefully acknowledged. Thanks are due to Norman

Harris and Dr. Pio Angelini for critical reviews of the manuscript.

Registry No. BHA, 25013-16-5; BHT, 128-37-0; SAN, 91-53-2; PG, 121-79-9; OG, 1034-01-1; DG, 1166-52-5; TOP, 128-37-0; EG, 831-61-8; TBHQ, 1948-33-0; Q, 117-39-5; MG, 99-24-1; HQ, 123-31-9; GA, 149-91-7; CA, 331-39-5; CHLA, 327-97-9; poly AO-79, 60837-57-2.

LITERATURE CITED

Abbot, J.; Waite, R. The Effect of Antioxidants on the Keeping Quality of Whole Milk Powder. I. Flavones, Gallates, Butylhydroxyanisole and Nordihydroquaiaretic Acid. *J. Dairy Res.* **1962**, *29*, 55–61.

Adamson, A. W. In *The Physical Chemistry of Surfaces*, 2nd ed.; Interscience Publications: New York, 1967; p 520.

Barclay, L. R. C.; Ingold, K. U. Autooxidation of a Model Membrane. Comparison of Egg Lecithin Phosphatidyl Choline in Water and in Chlorobenzene. *JAOCS, J. Am. Oil Chem. Soc.* **1980**, *102*, 7792–4.

Berner, D. L.; Conte, J. A.; Jacobson, G. A. Rapid Method for Determining Antioxidant Activity and Fat Stability. *J. Am. Oil Chem. Soc.* **1974**, *51*, 292–6.

Bieri, J. G.; Evarts, R. P.; Gart, J. J. Relative Activity of Alpha-Tocopherol and Gamma-Tocopherol in Preventing Oxidative Red Cell Hemolysis. *J. Nutr.* **1976**, *106*, 124–7.

Bunyan, J.; Green, J.; Edwin, E. E.; Diplock, A. T. Studies on Vitamin E. The Relative Activities of Tocopherols and Some Other Substances *in Vivo* and *in Vitro* Against Dialuric Acid-induced Haemolysis of Erythrocytes. *Biochem. J.* **1960**, *75*, 460–7.

Century, B.; Horwitt, M. K. Biological Availability of Various Forms of Vitamin E with Respect to Different Indices of Deficiency. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1965**, *24*, 906–16.

Chio, K. S.; Tappel, A. L. Synthesis and Characterization of the Fluorescent Products Derived from Malonaldehyde and Amino Acids. *Biochemistry* **1969**, *8*, 2821–6.

Chipault, J. R.; Mizuno, G. R.; Lundberg, W. O. The Antioxidant Properties of Spices. *Food Technol.* **1956**, *10*, 209–12.

Cillard, J.; Cillard, P. Behavior of Alpha, Gamma, and Delta Tocopherols with Linoleic Acid in Aqueous Media. *JAOCS, J. Am. Oil Chem. Soc.* **1980**, *57*, 39–42.

Cillard, J.; Cillard, P. Inhibitors of the Prooxidant Activity of Alpha Tocopherol. *JAOCS, J. Am. Oil Chem. Soc.* **1986**, *63*, 1165–9.

Corliss, G. A.; Dugan, L. R., Jr. Phospholipid Oxidation in Emulsions. *Lipids* **1970**, *5*, 846–53.

Cort, W. M. Hemoglobin Peroxidation Test Screens Antioxidants. *Food Technol.* **1974**, *28*, 60–66.

Cort, W. M. Antioxidant Properties of Ascorbic Acid in Foods. In *Ascorbic Acids: Chemistry, Metabolism, and Uses*; Seib, P. A., Tolbert, B. M., Eds.; Advances in Chemistry Series 200; American Chemical Society: Washington, DC, 1982; p 533 ff.

Daniels, D. G. H.; Martin, H. F. Antioxidants in Oats: Mono-esters of Caffeic and Ferulic Acids. *J. Sci. Food Agric.* **1967**, *18*, 589–95.

Dziedzic, S. Z.; Hudson, B. J. F. Phosphatidyl Ethanolamine as a Synergist for Primary Antioxidants in Edible Oils. *JAOCS, J. Am. Oil Chem. Soc.* **1984**, *61*, 1042–5.

Fukuzawa, K.; Tokumura, A.; Ouchi, S.; Tsukatani, H. Antioxidant Activities of Tocopherols on Fe^{2+}-ascorbate-induced Lipid Peroxidation in Lecithin Liposomes. *Lipids* **1982**, *17*, 511–3.

Furia, T. E.; Bellanca, N. The Properties and Performance of Poly AO™-79; a Nonabsorbable Polymeric Antioxidant Intended for Use in Foods. *J. Am. Oil Chem. Soc.* **1977**, *54*, 239–44.

Geissman, T. A. *The Chemistry of Flavonoid Compounds*; Pergamon Press: New York, 1962.

Griffith, W. C. *J. Soc. Cosmet. Chem.* **1954**, *5*, 249.

Guilbault, G. Fluorescence Analysis on Solid Surfaces. *Photochem. Photobiol.* **1977**, *25*, 403–411.

Gutteridge, J. M. C. The Measurement of Malondialdehyde in Peroxidized Ox-Brain Phospholipid Liposomes. *Anal. Biochem.* **1977**, *82*, 76–82.

Kaschnitz, R. M.; Hatefi, Y. Lipid Oxidation in Biological Membranes. Electron Transfer Proteins as Initiators of Lipid Oxidation. *Arch. Biochem. Biophys.* **1975**, *171*, 292–304.

Kikugawa, K.; Ido, Y. Studies on Peroxidized Lipids. V. Formation and Characterization of 1,4-Dihydropyridine-3,5-Dicarbaldehydes as Model of Fluorescent Compounds in Lipofuscin. *Lipids* **1984**, *19*, 600–8.

Koreh, K.; Seligman, M. L.; Demopoulos, H. B. The Effect of Dihydroergotoxine on Lipid Peroxidation in Vitro. *Lipids* **1982**, *17*, 724–6.

Kornbrust, D. J.; Mavis, R. D. Relative Susceptibility of Microsomes from Lung, Heart, Liver, Kidney, Brain and Testes to Lipid Peroxidation: Correlation with Vitamin E Content. *Lipids* **1980**, *15*, 315–22.

Kwon, T.; Watts, B. M. Malonaldehyde in Aqueous Solution and Its Role as a Measure of Lipid Oxidation in Foods. *J. Food Sci.* **1964**, *29*, 294–302.

Labuza, T. P. Kinetics of Lipid Oxidation in Foods. *CRC Crit. Rev. Food Technol.* **1971**, *2*, 355.

Lea, C. H. Antioxidant in Dry Fat Systems: Influence of the Fatty Acid Composition of the Substrate. *J. Sci. Food Agric.* **1960**, *11*, 143–50.

Lea, C. H; Ward, R. J. Relative Activities of the Seven Tocopherols. *J. Sci. Food Agric.* **1959**, *10*, 537–48.

Lew, Y. T.; Tappel, A. L. Antioxidant and Synergist Inhibition of Hematin-catalyzed Oxidative Fat Rancidity. *Food Technol.* **1956**, *10*, 285–9.

Lizada, M. C. C.; S. F. Yeng. Sulfite-Induced Lipid Peroxidation. *Lipids* **1981**, *16*, 189–94.

Marco, G. J. A Rapid Method for Evaluation of Antioxidants. *J. Am. Oil Chem. Soc.* **1968**, *45*, 594–8.

Moore, R. N.; Bickford, W. G. A Comparative Evaluation of Several Antioxidants in Edible Fats. *J. Am. Oil Chem. Soc.* **1952**, *29*, 1–4.

Morris, S. G.; Kraekel, L. A.; Hammer, D.; Myers, J. S.; Riemenschneider, R. W. Antioxidant Properties of the Fatty Alcohol Esters of Gallic Acid. *J. Am. Oil Chem. Soc.* **1947**, *24*, 309–11.

Olcott, H. S.; Einsett, E. An Antagonistic Effect with Antioxidants for Unsaturated Fats. *J. Am. Oil Chem. Soc.* **1958**, *35*, 159–60.

Porter, W. L. Recent Trends in Food Applications of Antioxidants. In *Autoxidation in Food and Biological Systems*; Simic, M. G., Karel, M., Eds.; Plenum Press: New York, 1980; pp 295–365.

Porter W. L. Method for Determining Oxidative Status of Unsaturated Lipids and Method for Evaluating Antioxidant Effectiveness in Lipid-polypeptide Layers. U.S. Patent 4,253,848, March 3, 1981.

Porter, W. L.; Wetherby, A. M.; Kapsalis, J. G. Fluorescence Test of Oxidative Abuse Status of Fats, Oils and Dry Whole Tissue. *J. Am. Oil Chem. Soc.* **1980**, *57*, 187A.

Porter, W. L.; Black, E. D.; Drolet, A. Use of Polyamide Oxidative Fluorescence Test on Emulsions to Evaluate Antioxidants in Wet Versus Dry Systems. Paper presented at the 182nd National Meeting of the American Chemical Society, New York, Aug 1981; AGFD No. 2.

Porter, W. L.; Black, E. D.; Drolet, A. M.; Kapsalis, J. G. Analytical Use of Fluorescence-Producing Reactions of Lipid- and Carbohydrate-Derived Carbonyl Groups with Amine End Groups of Polyamide Powder. In *The Maillard Reaction in Foods and Nutrition*; Waller, G. R., Feather, M. S., Eds.; ACS Symposium Series 215; American Chemical Society: Washington, DC, 1983; pp 47–70.

Pratt, D. E. Role of Flavones and Related Compounds in Retarding Lipid-Oxidative Flavor Changes in Foods. In *Phenolic, Sulfur and Nitrogen compounds in Food Flavor*; Charalambous, G., Katz, I., Eds.; ACS Symposium Series 26; American Chemical Society: Washington, DC, 1976; pp 1–13.

Pratt, D. E.; Miller, E. E. A Flavonoid Antioxidant in Spanish Peanuts. *JAOCS, J. Am. Oil Chem. Soc.* **1984**, *61*, 1064–7.

Sanderson, G. W.; Ranadive, A. S.; Eisenberg, L. S.; Farrell, F. J.; Simons, R.; Manley, C. H.; Coggon, P. Contribution of Polyphenolic Compounds to the Taste of Tea. In *Phenolic, Sulfur and Nitrogen Compounds* in *Food Flavor*; Charalambous, G., Katz, I., Eds.; ACS Symposium Series 26; American Chemical Society: Washington, DC, 1976; pp 4–46.

Sawicki, E.; Stanley, T. W.; Johnson, H. Direct Spectrophotofluorometric Analysis of Aromatic Compounds on Thin-Layer Chromatograms. *Microchem. J.* **1964**, *8*, 257–84.

Scott, J. W.; Cort, W. M.; Harley, H.; Parrish, D. R.; Saucy, G. 6-Hydroxychroman-2-carboxylic Acids: Novel Antioxidants.

J. Am. Oil Chem. Soc. **1974**, *51*, 200–3.
Sherwin, E. R. Antioxidants for Vegetable Oils. *J. Am. Oil Chem. Soc.* **1976**, *53*, 430–6.
Simpson, T. H.; Uri, N. Hydroxyflavones as Inhibitors of the Aerobic Oxidation of Unsaturated Fatty Acids. *Chem. Ind. (London)* **1956**, 956–7.
Sosulski, F.; Fleming, S. E. Chemical, Functional, and Nutritional Properties of Sunflower Protein Products. *J. Am. Oil Chem. Soc.* **1977**, *54*, 100A.
Takagi, T.; Iida, T. Antioxidant for Fats and Oils from Canary Seed. *JAOCS, J. Am. Oil Chem. Soc.* **1980**, *57*, 326–30.
Taylor, J. J.; Richardson, T.; Jasensky, R. D. Antioxidant Activity of Amino Acids Bound to Trolox-C. *JAOCS, J. Am. Oil Chem. Soc.* **1981**, *58*, 622–6.
Thompson, J. W.; Sherwin, E. R. Investigation of Antioxidants for Polyunsaturated Edible Oils. *J. Am. Oil Chem. Soc.* **1966**, *43*, 683–6.
Uri, N. *The Mechanism of the Oxidation of Linoleic Acid with Particular Reference to Metal Catalysis*; Fourth International Conference on the Biochemical Problems of Lipids; Butterworths: London, 1958; p 30.
Uri, N. Mechanism of Antioxidation. In *Autoxidation and Antioxidants*; Lundberg, W. O., Ed.; Interscience: New York, 1961; Vol. I, p 133 ff.
Weenen, H.; Porter, N. A. Autoxidation of Model Membrane Systems: Cooxidation of Polyunsaturated Lecithins with Sterols, Fatty Acids and Alpha-Tocopherol. *JAOCS, J. Am. Oil Chem. Soc.* **1982**, *104*, 5216–21.
Wenkert, E.; Fuchs, A.; McChesney, J. D. Chemical Artifacts from the Family Labiatae. *J. Org. Chem.* **1965**, *30*, 2931–4.
Wu, J. W.; Lee, M.-H.; Ho, C.-T.; Chang, S. S. Elucidation of the Chemical Structures of Natural Antioxidants Isolated from Rosemary. *JAOCS, J. Am. Oil Chem. Soc.* **1982a**, *59*, 339–345.
Wu, G.-S.; Stein, R. A.; Mead, J. F. Autoxidation of Phosphatidyl Choline Liposomes. *Lipids* **1982b**, *17*, 403–413.

Received for review April 5, 1988. Accepted November 9, 1988.